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Applied and Environmental Microbiology, Dec. 2009, p. 7326–7334 Vol. 75, No. 23
0099-2240/09/$12.00 doi:10.1128/AEM.01345-09

Novel Electrochemically Active Bacterium Phylogenetically Related to *Arcobacter butzleri*, Isolated from a Microbial Fuel Cell[▽]†

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Received 9 June 2009/Accepted 24 September 2009

Exoelectrogenic bacteria are organisms that can transfer electrons to extracellular insoluble electron acceptors and have the potential to be used in devices such as microbial fuel cells (MFCs). Currently, exoelectrogens have been identified in the *Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria*, as well as in the *Firmicutes* and *Acidobacteria*. Here, we describe use of culture-independent methods to identify two members of the genus *Arcobacter* in the *Epsilonproteobacteria* that are selectively enriched in an acetate-fed MFC. One of these organisms, *Arcobacter butzleri* strain ED-1, associates with the electrode and rapidly generates a strong electronegative potential as a pure culture when it is supplied with acetate. A mixed-community MFC in which ~90% of the population is comprised of the two *Arcobacter* species generates a maximal power density of 296 mW/liter. This demonstration of exoelectrogenesis by strain ED-1 is the first time that this property has been shown for members of this genus.

A microbial fuel cell (MFC) is a mimic of a biological system in which microorganisms transfer electrons from organic compounds to a conductive external electron acceptor under anaerobic conditions (6). In an MFC, the electron acceptor is provided by an artificial anode, which is connected to an electric circuit. Although the basic processes involved in the generation of electricity by bacteria have been known for many years, recent interest in MFC development has been stimulated by the need to find alternative, carbon-neutral sources of energy generation. MFCs are particularly useful for breakdown of organic matter in wastewater treatment plants, in which production of electricity as a by-product can be used to power the process or can be sold to offset the cost of operation (6). At present, although the key principles of MFC design and operation are well understood (19), the technical aspects and particularly the microbiological aspects (18) are still in development. Further optimization of the design and microbial composition of these devices is desirable as current MFCs achieve power densities of no more than 1,550 mW/liter (7), which limits their real-world applications (6).

The basic microbiological characteristics which influence the efficiency of an MFC are bacterial metabolism and bacterial electron transfer. Although most current MFCs perform optimally when they contain a mixed microbial community, some pure cultures that exhibit strong electrogenic activity in the MFC environment have been characterized (19). The electrogenic properties and some aspects of extracellular electron transfer have been defined for pure cultures of organisms such as *Geobacter sulfurreducens* (2, 3), *Escherichia coli* (27), *Shewanella putrefaciens* (15, 16), *Rhodoferax ferrireducens* (5), *Rhodopseudomonas palustris* DX-1 (40), and *Ochrobactrum anthropi* YZ-1 (41). The current list of confirmed exoelectrogens includes representatives of four of the five classes of *Proteobacteria* (only the *Epsilonproteobacteria* are not represented), as well as representatives of the *Firmicutes* and *Acidobacteria* (18). However, it is likely that novel electrogenic bacteria remain to be discovered.

The metabolic characteristics required for an electrogenic bacterium depend upon the specific application for which an MFC is used, because not all electrogenic bacteria are able to fully oxidize several substrates. For example, *Shewanella oneidensis* oxidizes lactate to acetate under anaerobic conditions, while *G. metallireducens* oxidizes acetate but not glucose (20). *R. ferrireducens* can oxidize acetate, lactate, and glucose but does not degrade ethanol, another common fermentation end product (11). For this reason, MFCs which are employed in wastewater treatment when complex compounds have to be degraded are often inoculated with a diverse microbial community (for example, methanogenic sludge [30]). Degradation of acetate is a key bacterial characteristic because acetate is a primary organic intermediate in the degradation of organic matter in anoxic aquatic sediments. Moreover, the ability to use artificial electron acceptors (anodic electrodes) provides bacteria such as *Geobacteraceae* with a competitive advantage over other microorganisms under such conditions. Analysis of the microbial community firmly attached to anodes harvesting electricity from a variety of sediments demonstrated that microorganisms in the family *Geobacteraceae* were highly enriched on these anodes (2, 35). Moreover, it was shown that an MFC initially inoculated with methanogenic sludge failed to consume acetate in the absence of anodic electrodes over a 1-year period (8).

Arcobacter spp. are inhabitants of human and animal hosts (14, 37) and also occur in various environments, including

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 2 October 2009.

wastewater (24), surface water (21), seawater (9), and groundwater (32). *Arcobacter* spp. belong to the *Epsilonproteobacteria*, which includes pathogens (e.g., *Campylobacter jejuni* and *Helicobacter pylori*), opportunistic pathogens, and nonpathogenic environmental isolates (4). Typically, these bacteria have genomes with low G+C contents (27 to 30%), although some *Epsilonproteobacteria*, such as *Wolinella* spp. and *Campylobacter curvus*, have higher G+C contents. The environmental bacteria group into four clusters: *Nautiliales*, *Arcobacter*, *Sulfurospirillum*, and *Thiovulgaceae*. The genus *Arcobacter* comprises *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter skirrowii*, and *Arcobacter cibarius*, all of which have been isolated from animals or food (particularly poultry), as well as *Arcobacter halophilus*, *Arcobacter nitrofigilis*, "*Candidatus* Arcobacter sulfidicus," and a number of species characterized so far only at the 16S rRNA gene level (4). A feature of both "*Ca*. Arcobacter sulfidicus" and *Arcobacter* sp. strain FWKBO is autotrophic metabolism under microaerobic conditions, in contrast to the heterotrophic growth of *A. butzleri*. Both of these organisms use oxidation of sulfide to sulfur and are obligate autotrophs. Some *Arcobacter* spp. may be capable of Mn and Fe reduction; isolates from Black Sea sediments (36) oxidized acetate in the presence of Mn oxide. This was the first evidence of Mn or Fe reduction in nitrate-reducing *Arcobacter* microaerophiles and nitrate reducers; previously, the only other epsilonproteobacterium identified with this ability was *Sulfurospirillum barnesii*. Thus, organisms related to *Arcobacter* comprise an ecologically significant new group of dissimilatory Fe- and Mn-reducing bacteria.

In the present study we isolated and characterized two strains phylogenetically related to *Arcobacter* spp. which are selectively enriched in an acetate-fed MFC. One of these strains, *A. butzleri* strain ED-1, which specifically associates with the MFC electrode, shows electrochemical activity when it is grown on acetate, and hence it is the first example of an exoelectrogenic epsilonproteobacterium.

MATERIALS AND METHODS

MFC. The MFC used in this work was a horizontal multielectrode bioelectrochemical reactor (8) in which immersed cathodes were replaced by semidry cathodes as described in patent application WO 2009/050513 A2 (V. V. Fedorovich, 23 April 2009). However, the structure of the liquid flow and the interelectrode distances were the same as those in the original design of this reactor. To avoid loss of biomass, the system was equipped with an external vessel and pump for liquid-phase recirculation. The total volume of the liquid phase was 1.5 liters.

Analysis and electrical measurements. The acetate concentration was determined using a Hewlett Packard gas chromatograph equipped with a glass column packed with Chromosorb 101 (80/100 mesh). The column, injector port, and flame ionization detector temperatures were set at 170°C, 180°C, and 190°C, respectively. Argon was used as a carrier gas. Before analysis, samples of influents and effluents were filtered using 0.45-μm membrane filters (ME 25; Schleicher & Schuell, Germany). Current and voltage were measured using a VersaSTATE 3 potentiostat (Princeton Applied Research, United States), and potentials were measured using an Ag/AgCl reference electrode.

MFC inoculation. The anodic zone of the cell was seeded with microorganisms from marine sediment from Cramond, Edinburgh, United Kingdom. The sediment was obtained at a depth of 3 m below the high-tide mark, but samples were obtained at low tide, when the sediment was exposed to the air, and at a temperature of ~10°C. Initially, 0.5 liter of marine sediment was diluted with 1.5 liters of a basal solution containing 0.05 M phosphate buffer (pH 5.7), 200 mg/liter $CaCl_2 \cdot 2H_2O$, 250 mg/liter $MgCl_2 \cdot 6H_2O$, and 500 mg/liter NH_4Cl; the low pH was used to select against methanogenic community members. Following decanting of the supernatant, basal medium that contained sodium acetate at a final concentration of 1.0 g chemical oxygen demand (COD)/liter was added. The fuel cell was filled with this final solution, the peristaltic pump was switched on, and the device was operated at room temperature (20°C). The hydraulic retention time of the liquid phase in the anodic zone of the MFC was 0.5 day. Sodium acetate in basal medium was added at approximately weekly intervals to maintain the total concentration at 1.0 g COD/liter.

Batch experiments in flasks. Flasks with a total volume of 500 ml were used for determination of electrogenic activity with and without an anodic electrode. In experiments in which the electrogenic activity of the MFC microbial community was investigated, 500 ml of the complete MFC liquid phase was added along with components of the basal medium at the standard concentrations. In experiments with pure cultures of *Arcobacter* spp., the flasks were filled with 500 ml of basal medium plus acetate, and a 2% inoculum of the relevant strain(s) grown microaerobically at 30°C in VanDamme (VD) medium (see below) was then added. When appropriate, sterilized reference (Ag/AgCl), counter (platinum), and working (carbon tissue) electrodes were introduced through sealed ports in the upper lid of the flask. The geometric surface of the working electrode was 120 cm^2. Flasks were incubated statically at room temperature (20°C).

Extraction of DNA from the MFC. Total community DNA was extracted from 100 μl of MFC supernatant, a 100-mg portion of the graphite electrode, or 100 mg of the original sediment inoculum for the MFC using a SoilMaster DNA extraction kit (Epicentre Biotechnologies) according to the manufacturer's instructions. DNA pellets were resuspended in 300 μl of sterile, distilled water.

16S rRNA gene amplification and sequencing. Almost full-length bacterial 16S rRNA gene fragments were amplified using primers complementary to positions 19 to 38 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1581 to 1541 (5′-AAGGAGGTGATCCAGCCGCA-3′) of the *E. coli* 16S rRNA gene as described previously by Fernández et al. (10). The PCR products were amplified using 50-μl reaction mixtures containing 10 μl of a community DNA sample or cells obtained directly from a plate colony, 0.5 μM each primer, 0.2 mM each deoxynucleoside triphosphate, 5 μl 10× *Taq* buffer (final concentration of $MgCl_2$, 1.5 mM), and 0.25 U of *Taq* DNA polymerase (Roche Applied Science). The PCR conditions were those employed by Fernández et al. (10), except that when amplification was done directly with cell colonies, an extra initial denaturation step (94°C for 10 min) was added prior to addition of the polymerase. PCR products were purified using a QIAquick PCR purification kit (Qiagen) prior to cloning or direct sequencing. Sequencing was performed with an ABI3730 capillary sequencer (The Gene Pool, School of Biological Sciences, University of Edinburgh) using the primers that were used for the initial PCR amplification for direct sequencing of PCR products or primers M13 forward (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse (5′-AACAGCTATGACCATG-3′) for fragments cloned into pCR2.1 (see below). 16S rRNA gene sequences were queried against the GenBank database using BLAST-N and were aligned with 16S rRNA gene sequences of related organisms using CLUSTAL-W. A neighbor-joining tree based on the alignment was constructed using the program MEGA4 (34). Confidence estimates for the branches in this tree were obtained by bootstrap resampling analysis with 1,000 replicates.

RFLP and DGGE analyses. Purified 16S rRNA gene PCR products (~1,500 bp) were digested with HaeIII and HhaI (New England Biolabs) and electrophoresed on 3.5% (wt/vol) MetaPhor agarose-Tris-borate-EDTA gels (Lonza Corporation) at 4 V/cm and 4°C for 17 h. Restriction fragment length polymorphism (RFLP) fragments for cloning were first separated on standard (0.8%) Tris-borate-EDTA-agarose gels and then extracted from these gels using a QIAquick gel purification kit (Qiagen). For denaturing gradient gel electrophoresis (DGGE), nested primers 341F-GC (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGGGAGGCAGCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′) were used to amplify the V3 variable region of the bacterial 16S rRNA gene from 1 μl of purified 1,500-bp first-round PCR product as described by Muyzer et al. (25). The PCR conditions used were initial denaturation at 95°C for 5 min, followed by 28 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1.5 min and then final extension at 72°C for 10 min. Ten-microliter samples of the extension products were loaded onto DGGE gels (22) containing a linear 30 to 70% denaturant gradient (100% denaturant was defined as 7 M urea [42%, wt/vol] and 40% [wt/vol] formamide). Electrophoresis was carried out with a DCode universal mutation detection system (Bio-Rad, Hertfordshire, United Kingdom) in 7 liters of 1× Tris-acetate-EDTA buffer at a constant temperature of 60°C for 960 min at 75 V. Silver staining of gels was carried out as described by Nicol et al. (26).

Cloning of 16S rRNA gene fragments. Purified fragments of amplified 16S rRNA genes obtained from RFLP gels or directly from PCRs were cloned into pCR2.1 using a TA cloning kit and chemically competent *E. coli* One Shot

INVαF′ (Invitrogen) according to the manufacturer's instructions. For fragments of restriction digests and PCR products which were not processed immediately, A tails were added to the DNA fragments using *Taq* polymerase prior to cloning. Plasmid DNA was isolated from positive clones using a QIAprep spin miniprep kit (Qiagen), and the presence of the insert that was the correct size was verified by digestion with EcoRI (New England Biolabs) prior to DNA sequencing.

Enumeration of bacteria from the MFC. Counting of viable bacteria was carried out using VD medium containing the following components (per liter): special peptone (Oxoid LP0072), 10 g; Lab Lemco powder (Oxoid L29), 5 g; yeast extract (Oxoid L21), 5 g; sodium glutamate, 2 g; sodium succinate, 2 g; $MgCl_2$, 1 g; and agar, 16 g. The *Arcobacter* selective medium (Houf-CNT) consisted of VD medium supplemented with 5% defibrinated horse blood and the following antibiotics: cefoperazone (16 μg/ml), novobiocin (32 μg/ml), and trimethoprim (64 μg/ml). Plates were incubated at 30°C either in an incubator, in gas jars under 5% (vol/vol) O_2-10% (vol/vol) CO_2, or in an anaerobic cabinet.

Bacteria attached to the carbon electrode were released by sonication. A 2-cm-by-2-cm piece of carbon cloth was cut out and put in a universal bottle with 10 ml of phosphate-buffered saline (PBS). Loosely attached bacteria were released by shaking the bottle at 150 rpm at room temperature for 10 to 20 min. The supernatant was removed, and the cloth was washed with 10 ml of PBS. Firmly attached cells were released by sonication in 10 ml of fresh PBS using a Heat Systems XL series sonicator at power setting 3 (amplitude, 15 to 20%) three times for 30 s. To enumerate bacteria, 10-fold serial dilutions in PBS were prepared, and 5-μl aliquots were plated onto both VD medium and Houf-CNT as described above.

Transmission and scanning electron microscopy. For transmission electron microscopy, *A. butzleri* ED-1 and *Arcobacter* sp. liquid phase, here and after referred to as *Arcobacter*-L, were grown in VD medium, washed twice in 1× PBS buffer, once in 0.5× PBS buffer, and once in sterile deionized water, and resuspended in sterile deionized water. The suspensions were spotted onto Formvar carbon-coated copper grids, air dried, and examined using a Philips CM120 BioTwin transmission electron microscope at an accelerating voltage of 70 kV. For scanning electron microscopy of aerobically grown ED-1, 1 ml of cells was grown overnight at 30°C in VD medium, washed in 1× PBS, resuspended in 200 μl of 1× PBS, and fixed with an equal volume of 3% glutaraldehyde; 20 μl of the suspension was then spotted onto a poly-L-lysine-coated coverslip. The samples were washed with 1× PBS, 0.5 × PBS, and deionized water and then sequentially dehydrated with three changes of 100% ethanol. Dehydrated samples were dried using a critical point dryer, sputter coated with Au/Pd alloy, and visualized with a BioSEM scanning electron microscope at an accelerating voltage of 5 kV. Square samples of a carbon electrode with attached ED-1 cells were fixed with 3% glutaraldehyde and then washed, dried, and visualized as described above.

Biochemical and growth characterization of *Arcobacter* spp. Biochemical properties of the isolated *Arcobacter* strains were determined using the API Campy strip identification system (BioMérieux, France) according to the manufacturer's instructions. Antibiotic resistance was assessed using the MASTRING-S antibiotic disk system (MAST Group Ltd., United Kingdom). To assess growth on particular carbon sources, a 2% inoculum of the relevant strain grown microaerobically at 30°C in VD medium was added to 10 ml of basal medium containing 1 g/liter of a carbon source, and the culture was incubated microaerobically at 30°C for 108 h. The optical density at 600 nm (OD_{600}) was determined at the beginning and at the end of the experiment. Growth rates of the *Arcobacter* strains on acetate were measured by adding 2% inocula grown microaerobically at 30°C in VD medium to 900-μl aliquots of basal medium with 1 g/liter acetate in a 48-well plate and incubating the plate microaerobically at room temperature (20°C).The OD_{600} was determined every 12 min for 45 h with a plate reader, and the means of the readings for five independent wells of each strain were used to generate the growth curves.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of *A. butzleri* ED-1 and *Arcobacter*-L determined in this study have been deposited in the GenBank database under accession numbers FJ968634 and FJ968635, respectively. The 16S rRNA gene sequences of other species cultured from the MFC have been deposited under accession numbers FJ968636 to FJ968639.

RESULTS

Acetate consumption and generation of potential in the MFC. Following inoculation with marine sediment obtained from Cramond, Edinburgh, the MFC was operated in recirculation mode while it was provided with an acetate minimal medium containing $CaCl_2$, $MgCl_2$, and NH_4Cl (see Materials and Methods). During continuous operation of the MFC, acetate was added to bring the concentration to the starting concentration (1.0 g COD/liter) at approximately weekly intervals. After 3 months of continuous operation, the maximal power density achieved by the MFC was 296 mW/liter. This level of power density was achieved largely as a result of the relatively low internal resistance (9 ± 0.5 Ω) of our device. To test the acetotrophic and electrogenic activity of the mature MFC microbial community, samples of the MFC liquid phase were taken and used to measure acetate consumption in flasks with a carbon anode and in flasks without a carbon anode. When an anode was present, acetate in the medium was rapidly consumed, indicating that there was efficient acetate-degrading activity in the established microbial community in the MFC (Fig. 1). This consumption of substrate was accompanied by a decrease in the anodic potential to approximately −400 mV. However, the acetate-degrading activity was much lower in the absence of an anode, indicating that the presence of an external electron acceptor was essential for efficient metabolism of this carbon source by the microbial community.

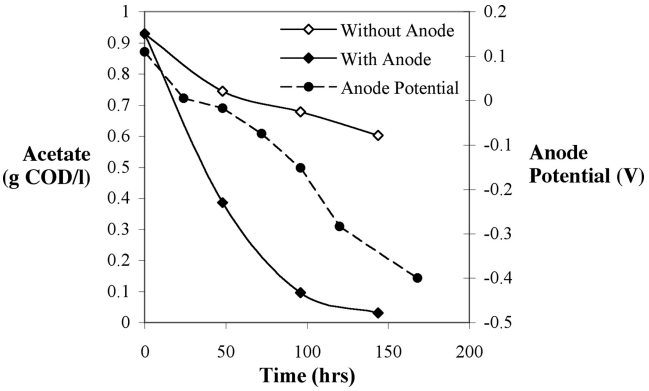

FIG. 1. Consumption of acetate (expressed in g COD/liter) in a flask containing the MFC liquid phase inoculated with a marine sediment microbial community following incubation with or without a carbon anode present. The development of the anodic potential over time with a carbon anode is also indicated.

Fingerprint analysis of bacterial diversity in the MFC. Samples of the liquid phase and graphite anode were taken from the operational, acetate-fed MFC following 4 weeks of continuous operation. Total community DNA was extracted from these samples and from the original marine sediment inoculum, and bacterial 16S rRNA gene fragments were amplified from the extracted DNA and analyzed by the RFLP method (Fig. 2A). This analysis showed that the samples were dominated by a few major species (bright bands) but that not all of these species were present in both the initial sediment and the MFC samples. In particular, a strong band at around 330 bp (Fig. 2A) was observed for the MFC samples but not for the environmental sample. As this band presumably represents a species which was selected for in the MFC environment, it was extracted from the electrode DNA sample in the gel, cloned, and sequenced. The sequence of the resulting 328-bp 16S rRNA gene fragment showed 100% identity to the 16S rRNA gene sequence of *A. butzleri* strain RM4018, an organism known to be an opportunistic human pathogen (23). Although

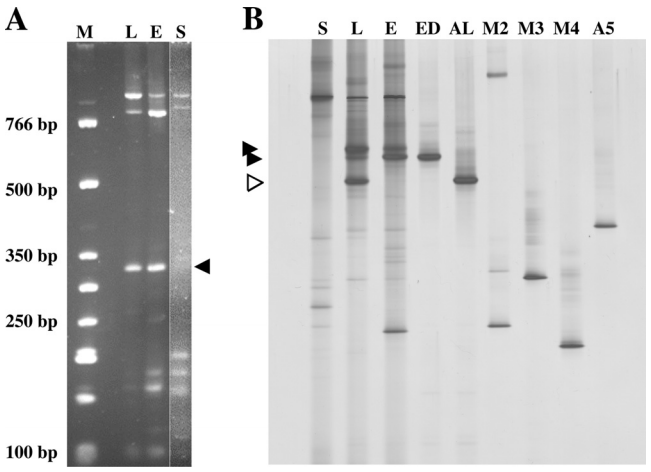

FIG. 2. (A) RFLP analysis of amplified, full-length bacterial 16S rRNA gene fragments from the MFC liquid phase (lane L), the carbon anode (lane E), and the original sediment inoculum (lane S). Lane S was overexposed to show the presence of faint bands and the absence of the 330-bp band specific to the MFC samples (arrowhead). Lane M contained size markers. (B) DGGE analysis of the amplified V3 variable region of bacterial 16S rRNA genes from the original sediment inoculum (lane S), the MFC liquid phase (lane L), and the carbon anode (lane E). Also shown are the DGGE profiles obtained for the cloned 16S rRNA genes of strain ED-1 (filled arrowheads) (lane ED) (amplification of 16S rRNA genes from genomic DNA of this species also gave rise to the upper band of the doublet) and the liquid-phase organism *Arcobacter*-L (lane AL) (open arrowhead). The gel also shows the DGGE profiles of 16S rRNA genes amplified from pure cultures of various species cultured from the MFC, including *Shewanella halitosis* (lane M2), *Acinetobacter* sp. (lane M3), *O. anthropi* (lane M4), and *Acinetobacter haeomlyticus* (lane A5) (see Table 2).

Arcobacter spp. have not previously been shown to be electrogenic, *Arcobacter* has been observed to be an unselected member of the community in a formate-enriched MFC (12). We also carried out DGGE fingerprint analysis of the V3 variable regions of bacterial 16S rRNA genes in the sediment and MFC samples, which was more sensitive to minor sequence variations in the 16S rRNA gene than RFLP analysis. The results (Fig. 2B) confirmed that the bacterial diversity in the mature MFC was quite different from that in the original sediment inoculum and also that there were differences between the electrode and liquid-phase samples. One prominent DGGE band for the liquid-phase sample (Fig. 2B) was not observed for the electrode sample, while a doublet of bands (Fig. 2B) was observed for both phases; none of these bands was detected when the initial sediment inoculum was examined.

Clone library analysis of bacterial diversity in the MFC. Small clone libraries (10 clones each) containing full-length 16S rRNA gene inserts were made using the same liquid- and electrode-derived community DNA samples that were used for RFLP analysis. Sequences from these clone libraries indicated that the electrode community was dominated by an RM4018-like *Arcobacter* strain (which we designated *A. butzleri* strain ED-1); 8 of 10 clones corresponded to this sequence (Table 1). However, analysis of the liquid-phase community revealed the presence of a different *Arcobacter* strain (*Arcobacter*-L). The two *Arcobacter*-like organisms share 95% sequence identity for 1,510 bp of the 16S rRNA gene (see Fig. S1 in the supplemental material). Again, *Arcobacter*-L dominated the liquid-phase community, and 8 of 10 clones corresponded to this organism (Table 1). The relatedness of the cloned 16S rRNA gene sequences of strain ED-1 and *Arcobacter*-L to 16S rRNA gene sequences of other members of the *Epsilonproteobacteria* is shown in Fig. 3. The 16S rRNA gene sequence of strain ED-1 was identical to that of strain RM4018, whose genome has been sequenced, and preliminary whole-genome sequencing of strain ED-1 (our unpublished data) showed that there was a high level of similarity and general colinearity of the two genomes. However, there were also significant genetic differences; for example, strain ED-1 lacked the sulfur oxidation and cytochrome *bd* oxidase genes.

The V3 regions of the strain ED-1 and *Arcobacter*-L 16S rRNA genes were amplified from the corresponding clones and analyzed alongside the mixed-community samples by DGGE (Fig. 2B). The ED-1 clone produced the lower band of the prominent doublet seen in both the electrode and liquid-phase communities, confirming that ED-1 is the main electrode-associated species in the MFC and indicating that this organism is also present in the liquid phase. DGGE of ED-1 genomic DNA-derived PCR products also produced the upper band of this doublet (data not shown), suggesting that the doublet is a PCR-DGGE artifact derived from the ED-1 16S rRNA gene sequence. The *Arcobacter*-L sequence corresponded to the additional major band found in the liquid-phase community, which again is consistent with its distribution in the clone libraries.

The remaining two clones obtained from the electrode and

TABLE 1. 16S rRNA gene clones obtained from the electrode and liquid-phase samples

Sample	Clone(s)	Closest database match	Accession no.	Length of overlap (nucleotides)	% Identity
Electrode	1–4, 6, 7, 9, 10	*A. butzleri* RM4018	CP000361	1,509	100
	5	Uncultured *Acinetobacter* sp. clone TCCC 11087[a]	EU567041	326	100
	8	*Shewanella* sp. strain S4[a]	FJ589031	310	99
Liquid phase	1, 3, 5–10	Uncultured *Arcobacter* sp. clone DS118	DQ234201	1,512	98
	2	*A. butzleri* RM4018[b]	CP000361	1,150	100
	4	Uncultured bacterium mle1-2 (*Bacteroides* sp.)[b]	AF280841	1,415	95

[a] Non-*A. butzleri* ED-1 clones obtained from the electrode sample were all chimeric with ED-1-derived sequences. The approximate length of the non-ED-1 portion of the sequence is indicated by the length of overlap.

[b] Non-*Arcobacter*-L clones obtained from the liquid sample were all chimeric with *Arcobacter*-L-derived sequences. The approximate length of the non-*Arcobacter*-L portion of the sequence is indicated by the length of overlap.

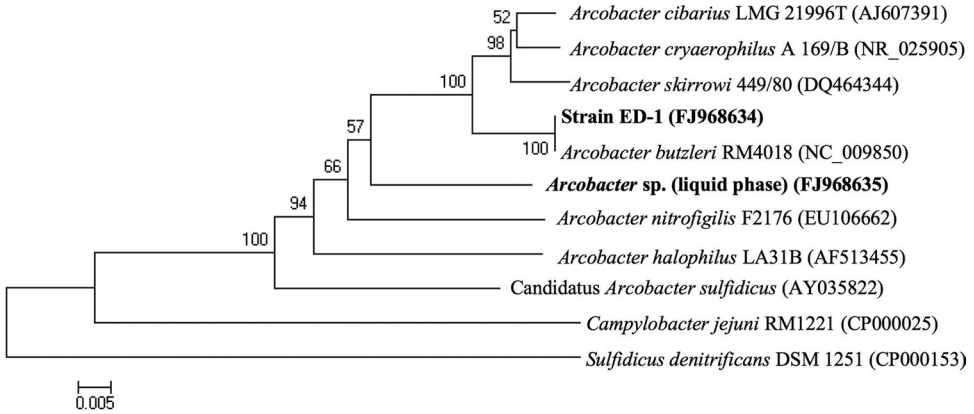

FIG. 3. Phylogenetic tree based on full-length 16S rRNA gene sequences for strain ED-1 and *Arcobacter*-L (bold type) and various other members of the *Epsilonproteobacteria*. Accession numbers for the sequences are indicated in parentheses. The tree was constructed using the neighbor-joining method; the bootstrap values at the nodes were calculated using 1,000 replicates. Bar = 0.5% sequence divergence.

liquid-phase samples were chimeric, a common feature of 16S rRNA gene-derived clone libraries, particularly when they are dominated by one sequence (38, 39). For the electrode sample, the chimeras consisted of sequences derived from an uncultured *Acinetobacter* species and *Shewanella* sp. strain S4 fused to the ED-1 sequence. One chimera from the liquid-phase sample consisted of an uncultured *Bacteroides* sequence fused to the *Arcobacter*-L sequence; the second chimera was a fusion between the 16S rRNA gene sequences of ED-1 and *Arcobacter*-L (Table 1).

Enumeration and isolation of bacteria from the MFC by plate culturing. To isolate species from the MFC as pure cultures, a liquid sample was taken from the MFC after it had been operated for 3 months. Bacteria from this sample were enumerated on both VD medium and Houf-CNT medium as described in Materials and Methods. Three different growth atmospheres were used: aerobic, microaerobic, and anaerobic. Aerobic cultivation was in air in a standard bacteriological incubator, microaerobic growth was in gas jars containing 5% (vol/vol) O_2 and 10% (vol/vol) CO_2, and anaerobic growth was in a miniMACS anaerobic workstation. Despite the low redox potential of the culture medium, the bacteria isolated from the medium were mainly aerophiles or aerotolerant organisms and microaerophiles rather than anaerobic bacteria. The numbers of bacteria isolated from VD medium were similar whether acetate (20 mM) or glucose (10 mM) was added as an additional carbon source. The viable counts were 4.6×10^7, 4.5×10^7, and 2.1×10^4 CFU/ml for aerobic, microaerobic, and anaerobic growth, respectively.

Single-colony purification and replating allowed isolation of both strain ED-1 and *Arcobacter*-L from the plates, and the 16S rRNA gene sequences of these organisms were determined to be consistent with those shown in Fig. S1 in the supplemental material by direct PCR amplification and sequencing from colonies. It was estimated by plate counting and microscopy that ~90% of the viable organisms in the sample were these species, confirming that they had been stably maintained in the MFC during its additional 2 months of operation. However, we also isolated two *Acinetobacter* spp. from the culture plates, one of which had a 16S rRNA gene sequence identical to that obtained in our previous clone analysis, as well as an additional *Shewanella*-like organism (which grew only microaerobically) and an *Ochrobactrum* species (Table 2). DGGE of the *Shewanella* (isolate M2) 16S rRNA gene indicated that it was not a major species in the MFC operated for 1 month but was detectable in the original sediment (Fig. 2B); it may have proliferated in the MFC during the additional 2 months of operation prior to culturing. While the *Ochrobactrum* sp. (isolate M4) was not detectable in any of the community samples based on the DGGE profile, the two *Acinetobacter* spp. (isolates M3 and A5) were detectable in both the MFC liquid-phase and electrode communities.

TABLE 2. 16S rRNA gene sequences of non-*Arcobacter* colonies isolated by plating from the liquid-phase sample

Plate	Isolate	Closest database match	Accession no.	Length of overlap (nucleotides)	% Identity
Aerobic	3	Uncultured *Acinetobacter* sp. clone VE12D01[a]	GQ179716	1,405	99
	4	*Ochrobactrum* sp. strain c261	FJ950617	655	100
	5	*Acinetobacter haemolyticus*	AY047216	1,421	99
Microaerobic	2	*Shewanella* sp. strain JC 19[b]	FM887036	1,342	99
	3	Uncultured *Acinetobacter* sp. clone VE12D01[a]	GQ179716	1,428	99
	4	*Ochrobactrum* sp. strain c261	FJ950617	655	100

[a] The sequence of aerobic isolate 3 and microaerobic isolate 3 (*Acinetobacter* sp.) is identical to that obtained for clone 5 from the electrode DNA sample (Table 1).

[b] The sequence of microaerobic isolate 2 (*Shewanella*) is only 91% identical in a 207-nucleotide overlap with that obtained for clone 7 from the electrode DNA sample (Table 1).

TABLE 3. Characteristics of strain ED-1, *Arcobacter*-L, and *A. butzleri* RM4018[a]

Characteristic	Strain ED-1	*Arcobacter*-L	*A. butzleri* RM4018[b]
Cell shape	Vibrioid	Vibrioid or small rod	Vibrioid
Motility	++	+	++
Microaerobic growth	++	++	++
API Campy profile	6 401 130	6 401 130	6 401 130
Alkaline phosphatase	+	+	+
Assimilation of:			
Glucose	−	−	−
Succinate	+	+	+
Acetate	+	+	+
Propionate	+	+	+
Malate	−	−	−
Citrate	−	−	−
Antibiotic resistance			
Ampicillin	R	R	R
Cefoperazone	R	R	R
Gentamicin	S	S	S
Kanamycin	S	S	S
Novobiocin	R	R	R
Streptomycin	S	S	S
Trimethoprim	R	R	R
Growth on C sources			
Acetate	+	±	−
Lactate	+	+	+
Succinate	+	±	ND
Fumarate	±	+	+
Glutamate	±	±	ND

[a] R, resistant; S, sensitive; ND, not determined.
[b] Antibiotic resistance and C source utilization data for *A. butzleri* RM4018 were obtained from reference 23.

Characterization of the two *Arcobacter* species. Microscopic analysis, biochemical tests, and antibiotic resistance profiles were used to differentiate the two *Arcobacter* species and compare them to *A. butzleri* RM4018. Transmission electron microscopy of cultures grown in VD medium showed that the larger, more vibrioid cells of strain ED-1 apparent when light microscopy was used (Table 3) may have been due to cell division that was more active than that of *Arcobacter*-L (Fig. 4A and B). Both strains appeared to have appendages when they were examined by transmission electron microscopy, and scanning electron microscopy of ED-1 cells (Fig. 4C) showed that they had lateral and branched filaments, as well as clear polar flagella. Scanning electron microscopy of ED-1 attached to the carbon electrode (Fig. 4D) showed no such appendages; further work is required to determine whether these structures are nanowires involved in extracellular electron transfer. The biochemical properties and antibiotic resistance profiles of these isolates were characterized using the API Campy system and Mastrings antibiotic disks, respectively. The API Campy numerical profiles for strain ED-1 and *Arcobacter*-L were identical. Both strains were able to metabolize succinate, propionate, and acetate but not malate, citrate, or glucose, and they also had similar antibiotic resistance profiles (Table 3). Growth on the MFC minimal medium supplemented with a variety of compounds as sole carbon sources was assessed microaerobically over a 108-h period (Table 3). The data showed that while

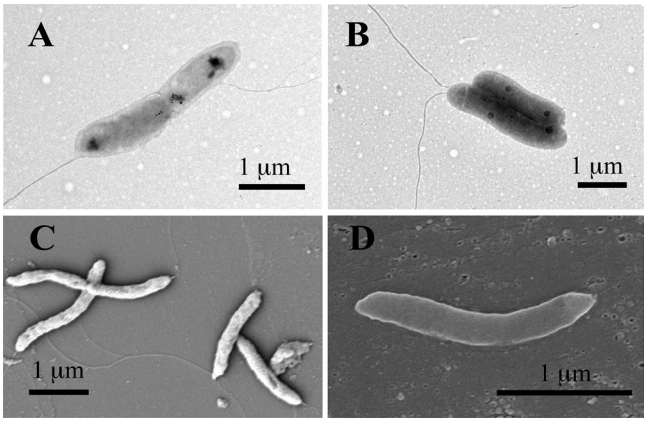

FIG. 4. (A and B) Transmission electron micrographs of strain ED-1 (A) and *Arcobacter*-L (B) following growth in VD medium. (C) Scanning electron micrograph of aerobically grown ED-1 cells, showing polar and branched appendages. (D) Scanning electron micrograph of an ED-1 cell attached to a carbon electrode following growth on acetate.

both *Arcobacter* strains grew well on lactate, strain ED-1, but not *Arcobacter*-L, showed significant growth on acetate; the reverse was true for growth on fumarate.

As acetate was the sole carbon source in the MFC from which these organisms were isolated, the growth of both *Arcobacter* strains on this compound was analyzed in more detail by growing multiple replicate cultures of each strain in a plate reader for 45 h (Fig. 5). The mean OD_{600}s obtained in this experiment showed that strain ED-1 grew rapidly on the acetate medium to an OD_{600} of >0.1, while *Arcobacter*-L showed much slower growth and the final OD_{600} was lower. This is consistent with the observation that ED-1 was the main acetotrophic species in the operational fuel cell and that ED-1, but not *Arcobacter*-L, was associated with the electrode. It is possible that *Arcobacter*-L persisted in the liquid phase of the MFC due to utilization of compounds produced by ED-1 and/or other members of the microbial community.

Electrogenic activity of strain ED-1. To test the electrogenic activity of a pure culture of strain ED-1, sterilized

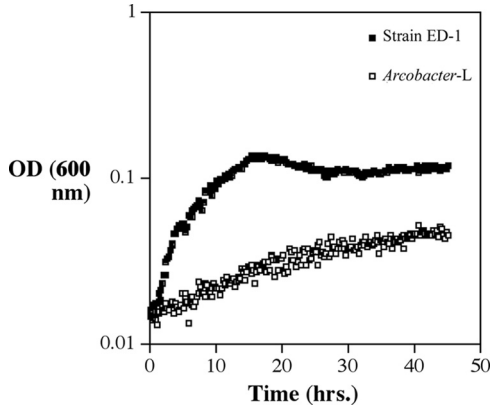

FIG. 5. Growth of strain ED-1 and *Arcobacter*-L on acetate minimal medium under microaerobic conditions in a microtiter plate at room temperature (20°C). The OD_{600} was measured every 12 min, and the values are the mean OD_{600}s for five independent culture wells.

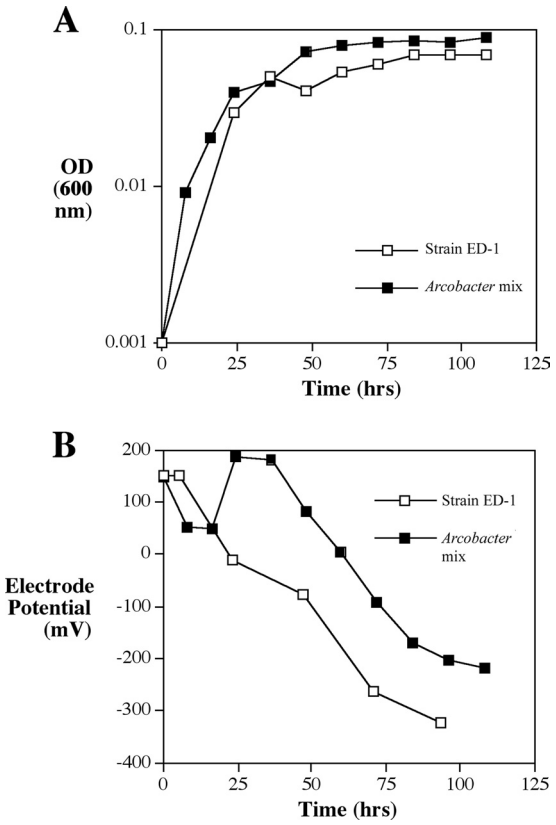

FIG. 6. (A) Growth of strain ED-1 and a culture started with a 1:1 inoculum of strain ED-1 and *Arcobacter*-L in acetate minimal medium in a sealed culture flask containing a sterilized, unconnected carbon electrode. (B) Electrode potentials at the carbon electrode in flasks containing the acetate-grown culture of strain ED-1 and the acetate-grown culture started with a 1:1 inoculum of strain ED-1 and *Arcobacter*-L shown in panel A.

flasks containing acetate minimal medium and a graphite electrode were inoculated with this organism or a 1:1 mixture of ED-1 and *Arcobacter*-L, sealed, and incubated at room temperature for 4 to 5 days. The growth of the bacteria and the anodic potential were monitored throughout this period (Fig. 6). Although the growth of the pure cultures on acetate medium under these conditions was similar to the growth of ED-1 in the absence of an electrode, significantly negative anodic potentials (−200 to −300 mV) were obtained in both ED-1 and mixed cultures within 4 days. Consistent with its superior growth on acetate and its association with the electrode, ED-1 was the dominant species in the mixed culture after 5 days, although the electrode potential obtained with the mixed culture was slightly less negative than that obtained with the ED-1 pure culture. These data confirm that *A. butzleri* strain ED-1 was the primary electrogenic species in the mature MFC.

DISCUSSION

Using a multielectrode horizontal MFC device inoculated with marine sediment, we isolated a novel *A. butzleri* strain which selectively proliferates on acetate in this environment and acts as a potent exoelectrogen. Transfer of the original sediment community to the MFC would have selected for an ability to utilize acetate and to transfer electrons to the anode, as well as an ability to proliferate at room temperature and at low pH. Our approach, which involved allowing the microbial community to adapt to this environment for 4 weeks and then characterizing the dominant microbial species present by culture-independent molecular methods, avoided having to use culture techniques to isolate electrogenic bacteria. Typically, exoelectrogens have been isolated from MFCs using agar plates containing soluble Fe(III) citrate (28), Fe(III) pyrophosphate (29), or insoluble Fe(III) oxide (13) as an electron acceptor, but these Fe(III) plating methods do not directly select for electricity-producing bacteria and may exclude electrogenic organisms which cannot respire using iron (41). Nutrient agar plates can also be used to isolate electrogens from MFCs (30), but the lack of selection with these methods allows nonelectrogenic bacteria to grow equally well. By first identifying the dominant species present in our MFC using culture-independent methods, we were able to isolate these species in pure culture on appropriate general rich (VD) and selective (Houf-CNT) media, along with other minor members of the microbial community. The validity of our approach was demonstrated by the observation that an estimated ~90% of the bacterial cells in the MFC as determined by plate counting and microscopy were cells of the species initially detected by our molecular techniques.

The mature, acetate-fed MFC community composed of ~90% *A. butzleri* strain ED-1 and *Arcobacter*-L cells proved to be highly efficient for generation of electricity. During continuous operation the fuel cell achieved a maximal power density of 296 mW/liter, which, although lower than the highest reported MFC power density (1,550 mW/liter for a bicarbonate buffer-containing fuel cell [7]), compares very favorably with the values reported previously for acetate-fed MFCs, 48 mW/liter (31) and 12.7 mW/liter (17). However, a portion of the difference can be attributed to electrode design and internal resistance rather than to the colonizing species. The same microbial community was able to consume acetate efficiently when an anode that acted as an artificial electron acceptor was present and to generate a strong electronegative potential (−400 mV) at the anode. However, although *Arcobacter*-L was the major planktonic component of this community, its role in acetate utilization and generation of electricity seems to be secondary to the role of the electrode-associated strain ED-1. Pure cultures of *Arcobacter*-L do not grow well on acetate, and a 1:1 mixture of *Arcobacter*-L and ED-1 inoculated into an electrode-containing flask and fed acetate rapidly becomes dominated by ED-1 and generates an electrode potential no more electronegative than that of a pure ED-1 culture. The persistence of *Arcobacter*-L in the MFC liquid-phase community may therefore be due to syntrophic interactions with other minor members of the microbial community and/or specific features of the MFC environment. In contrast, pure ED-1 cultures grow well on acetate and generate significant electronegative potentials in the presence of a carbon anode. *A. butzleri* strain ED-1 is therefore a potentially useful exoelectrogen for acetate-fed fuel cells.

It is of interest that, unlike the results for other enrichments of electrogenic, acetotrophic bacteria from marine sediments (2), no *Geobacter* spp. were isolated in our study. This may in

part be due to the low pH (pH 5.7) used for our MFC medium, compared to the higher pH (pH 6.8) generally used in *Geobacter*-containing fuel cells (3). Consistent with this hypothesis, in a formate-fed MFC in which *Arcobacter* sp. coexists with *Geobacter* sp. on the electrode, the pH of the medium is 7.0 (12). It is also possible that a low but nonzero level of oxygen in our system may have given the microaerophilic *Arcobacter* spp. an advantage over the strictly anaerobic *Geobacter* cells. Oxygen was not purged from the system following setup, and a small amount of oxygen would have been reintroduced during sampling. Consistent with this interpretation, the plate counts for liquid samples from the MFC were higher for aerophiles and microaerophiles than for anaerobes. The aerophiles may be instrumental in consuming oxygen in the system, thus maintaining the anode itself in a fully anaerobic environment and assisting electrogenesis.

As is typical for *Campylobacter* and *Arcobacter* spp. (23), strain ED-1 is unable to use sugars, such as glucose, as carbon sources. Analysis of the glycolytic pathway using the genome of *A. butzleri* RM4018 and RAST (1) indicated that the first step involving glucokinase is deficient but all other glycolytic enzymes catalyzing the formation of pyruvate are present. Our results differ slightly from those reported previously (23) in that strain ED-1 grew on acetate and propionate, both in microtiter plates and in an API Campy test kit. As a control we also tested *A. butzleri* RM4018 (= ATCC 12481) with the API Campy kit and found that this strain grew on both acetate and propionate (Table 3). However, the growth was more limited than the growth when the organism was grown in amino acid-containing media. In agreement with these data, we found that there was growth on lactate but not on citrate; the lack of growth on citrate may have been due to the absence of a citrate transporter. The inability to utilize glucose has also been found in other exoelectrogens, such as *G. sulfurreducens* (2), but strain ED-1 has an advantage over other species, such as *S. putrefaciens*, which cannot oxidize acetate under anaerobic conditions (33). Because acetate is the primary organic intermediate in a number of organic degradation pathways, its oxidation is an important step in accessing the full energetic potential of a variety of potential substrates, such as those found in wastewater samples. Thus, even if ED-1 is found not to be able to metabolize directly all the compounds present in a particular MFC substrate, it can potentially be a useful organism to add to MFCs utilizing such substrates in order to facilitate the efficient transfer of electrons from the acetate intermediate to the anode.

Our demonstration of electrogenic properties associated with the genus *Arcobacter* is novel and expands the range of microbial phyla which are known to act as exoelectrogens to include the *Epsilonproteobacteria*. There was one previous report of isolation of *Arcobacter* sp. from an MFC; Ha et al. (12) showed that a major DGGE band detected for DNA from the electrode of an MFC fed with acetate or formate gave rise to 16S rRNA gene clones with homology to *A. butzleri*, *A. skirrowii*, or *A. cibarius*. Surprisingly, the same DGGE band was equally prominent in the sediment inoculum used to initiate the MFC community, as were several other bands in the community profile. This suggests that in this system the selection pressure in the MFC environment was not as strong as that in our system, perhaps because the starting sample was derived from a sewage treatment plant rather than from natural sediments. Hence, *Arcobacter* spp. are not specifically enriched in this MFC environment; these authors speculated that *Arcobacter* spp. might persist in the MFC due to their preference for microaerobic environments and their ability to utilize formate, while they do not participate directly in electricity production (12). In contrast, our RFLP and DGGE analyses indicated that the two *Arcobacter* species that we isolated were not detectable in our original sediment inoculum but were the dominant species after 4 weeks of operation of the acetate-fed MFC. Therefore, strong selection for their enrichment in this environment was evident. In the case of strain ED-1, this was probably because it was able to utilize acetate in the medium as a carbon source and the graphite electrode as an electron acceptor more efficiently than other species in the initial inoculum and was also well adapted to other aspects of the MFC environment. Our community analysis also detected other species more typically associated with MFCs, such as *Shewanella* sp. (16) and *O. anthropi* (41) along with *Acinetobacter* sp., which were previously suggested to be involved in acetate consumption in the *Arcobacter*-containing MFC (12). However, DGGE analysis showed that, unlike the *Arcobacter* species, these additional species were not major members of the electrode-associated or liquid-phase communities in our MFC.

Arcobacter spp. include both environmental nonpathogens and opportunistic human pathogens. There is a growing list of opportunistic human-pathogenic bacteria which can act as exoelectrogens in an MFC, including *O. anthropi* YZ-1 (41) and *Pseudomonas aeruginosa* (30). *P. aeruginosa* can transfer electrons to an electrode via soluble mediators; the exocellular electron transfer mechanisms of *O. anthropi* YZ-1 remain to be determined. The closest relative of strain ED-1 is *A. butzleri* RM4018, which is considered an opportunistic pathogen. Although there is no current evidence of pathogenicity of strain ED-1, it has been speculated (41) that the production of exopolysaccharides by pathogenic species may help these organisms colonize MFC electrodes. *A. butzleri* RM4018 is known to produce lipooligosaccharides via a locus that is conserved among other *A. butzleri* strains (23); these molecules may be involved in the attachment of the environmental isolate strain ED-1 to the MFC electrode, but their role in the lifestyle of *Arcobacter* spp. has not been examined. Likewise, we need to understand more about electron transfer by strain ED-1. It will be interesting to examine whether the lateral appendages produced by ED-1 are involved in electron transfer by examining their ability to reduce silica ferrihydrite in vitro. Attempts to answer these questions and to determine the genetic basis of utilization of acetate and other carbon sources by ED-1 should also be aided by our ongoing genomic analysis of this strain.

ACKNOWLEDGMENTS

We thank The Gene Pool, School of Biological Sciences, University of Edinburgh, for DNA sequencing, Lucas Black for assistance with the plate reader experiments, Denis Trubitsyn for help with transmission electron microscopy, and John Hopkins for excellent technical assistance with the bacteriological testing.

This work was funded by a project grant from the Leverhulme Trust (grant F/00 158/BX) to A.F., who was supported by a fellowship from the Darwin Trust of Edinburgh; by a studentship from the Medical Research Council (United Kingdom) to M.C.K.; and by

award IKTF 4P-2038 from the School of Informatics, University of Edinburgh, to I.G.

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